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Hydrogen Sulfide in Preeclampsia

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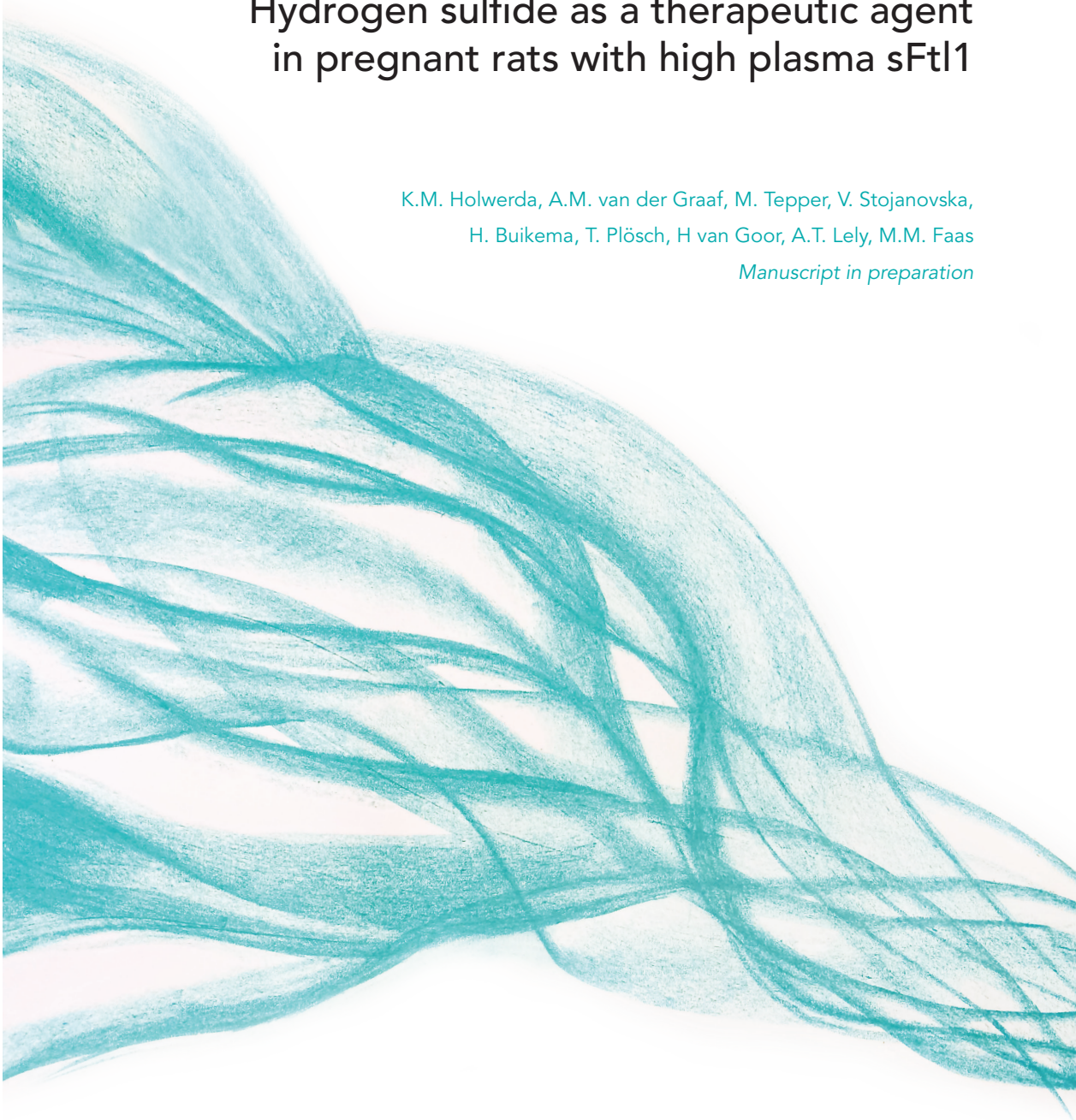
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Chapter 6

Hydrogen sulfide as a therapeutic agent in pregnant rats with high plasma sFtl1

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Abstract

Hydrogen sulfide (H₂S) is a gaseous regulator in the vasculature. It regulates blood pressure, vessel growth, modulates the immune system, and scavenges reactive oxygen species. Previously, we observed that H₂S attenuated hypertension and proteinuria in rats induced by overexpression of soluble FMS-like tyrosine kinase 1 (sFlt1), a strong anti-angiogenic factor. As high levels of sFlt-1 during pregnancy is used as a model for preeclampsia, the aim of the present study was to evaluate whether H₂S might have therapeutic effects on blood pressure and proteinuria in pregnant rats with high sFlt1.

The safety of NaHS (an H₂S-donor) was evaluated by analyzing maternal blood pressure and fetal characteristics in pregnant Sprague Dawley rats. Rats were injected (twice daily; IP) with vehicle (0.9% NaCl), 50 µmol/kg NaHS, or 200 µmol/kg NaHS from day 12 until day 19 of gestation. Thereafter, the therapeutic effect of NaHS was evaluated in pregnant Sprague Dawley rats with high sFlt1, induced by adenoviral sFlt1 overexpression. High sFlt1 rats were IP injected from day 12 until day 19 with either 0.9% NaCl or 100 µmol/kg NaHS per day. Control pregnant rats received adenovirus expressing an empty vector and were treated with 0.9% NaCl. Urinary albumin excretion was measured on day 17. At sacrifice at day 19, blood pressure was measured and plasma sFlt1 was determined. Glomerular lesions, renal macrophage infiltration, and α -smooth muscle actin expression was analyzed using immunohistochemistry. Endothelial function and angiotensin II sensitivity of rat thoracic aortic rings was studied *ex vivo*. Renal mRNA expression of *Vegfa* and the angiotensin receptor 1 and 2 (*At1r*, *At2r*) were evaluated using real-time RT-PCR.

In the safety-study, no harmful effects of NaHS on both fetal and maternal parameters were observed. NaHS treatment resulted in increased fetal weights without affecting placental weight. In rats with high sFlt1, NaHS restored the fetal brain/liver ratio. Because pregnant rats with high sFlt1 did not develop hypertension or albuminuria, we were not able to assess the effect of NaHS on these two parameters. High sFlt1 induced glomerular lesions, glomerular and interstitial macrophage infiltration, and prefibrotic lesions, which were not affected by NaHS treatment. NaHS treatment attenuated sensitivity to angiotensin II in thoracic aorta rings and increased renal *At2r* mRNA.

Although high levels of sFlt1 induced glomerular lesions, renal macrophage infiltration and prefibrotic kidney injury, in the present study, high sFlt1 did not induce endothelial dysfunction, hypertension and proteinuria. Therefore, we were not able to answer our

main study question whether H₂S attenuates hypertension and proteinuria in pregnant rats. However, the beneficial effects of H₂S on fetal growth possibly indicating increased placental blood flow. Moreover, as increased angiotensin II sensitivity is a major feature during human preeclampsia, the ability of NaHS to reduce the sensitivity might be an interesting target for H₂S-therapy to further evaluate.

Introduction

Hydrogen sulfide (H₂S) is an endogenous gaseous signaling molecule, acknowledged as the third gasotransmitter besides nitric oxide (NO) and carbon monoxide (CO).¹ A convincing line of evidence has shown that H₂S is an important modulator of endothelial homeostasis.² H₂S is involved in regulation of the vascular tone,³⁻⁵ inhibition of platelet adhesion,⁶ and promotion of new vessel growth.⁷ Moreover, H₂S protects the endothelium from inflammation by direct modulation of the immune system,⁸ and by local scavenging of free radicals.⁹ H₂S related therapies have shown beneficial effects in several experimental models of (cardiovascular) diseases, such as attenuating angiotensin II induced renal damage and hypertension in rats,¹⁰ increasing angiogenesis in rats with hindlimb ischemia,¹¹ and protecting against myocardial ischemia.¹²

Soluble FMS-like tyrosine kinase 1 (sFlt1) is a splice variant of the vascular endothelial growth factor (VEGF) receptor, lacking the transmembrane and cytoplasmic domains.^{14,15} By antagonizing VEGF, sFlt1 can inhibit VEGF signaling in the vasculature,¹⁵⁻¹⁷ causing endothelial dysfunction, decreased angiogenesis, and impaired capillary repair.^{18,19} Elevated levels of sFlt1 have been associated with endothelial dysfunction in several diseases, such as vasculitis and chronic kidney disease.^{20,21} Recently, we have shown that H₂S has beneficial effects in a rat model with impaired angiogenesis, induced by high levels of sFlt1.¹³ H₂S attenuated hypertension, proteinuria and renal lesions.¹³ Thus, H₂S might be an attractive molecule in counteracting the adverse effects of sFlt1 on the endothelium.

High levels of sFlt1 have been found in women with preeclampsia, a pregnancy-specific syndrome which is characterized by hypertension, albuminuria, endothelial dysfunction, and glomerular disease, i.e. endotheliosis.^{19,22-24} In women with preeclampsia,

elevated levels of plasma sFlt1 are thought to contribute to the onset of clinical symptoms.¹⁹ Indeed, pregnant rats, in which high levels of sFlt1 were induced, developed hypertension, albuminuria and glomerular endotheliosis.¹⁹ We thus hypothesized that H₂S might have therapeutic potential in pregnant rats with high sFlt1, too. To test this hypothesis, pregnant rats were injected with an adenovirus expressing sFlt1 (or control virus) on day 8 of pregnancy, and subsequently treated with NaHS or vehicle from day 12. Besides the effects of H₂S on blood pressure, albumin excretion and renal lesions, we also tested the effect of H₂S on endothelial function and sensitivity for angiotensin II in this model. As H₂S therapy was never applied in pregnant rats before, we first evaluated the safety of H₂S in healthy pregnant rats.

Methods

Experimental design

In this study, the *in vivo* therapeutic effect of H₂S on pregnant rats with high plasma sFlt1 was investigated. Before starting this study, we performed a safety-study in which we treated healthy pregnant rats twice daily with two different concentrations of the H₂S-donor sodium hydrosulfide (NaHS, Sigma, St. Louis, MO, USA). In order to determine whether the use of NaHS had adverse effects on rat pregnancy, we analyzed the effect on maternal blood pressure, and other characteristics such as fetal weight and gross anomalies, placental weight and fetal resorption ratio.

We found no adverse effects of NaHS on pregnancy, therefore, we continued with testing the therapeutic effect of H₂S in a rat model with high plasma sFlt1. The experimental setup consisted of three groups; healthy pregnant rats treated with vehicle, pregnant rats overexpressing sFlt1 treated with vehicle, and pregnant rats overexpressing sFlt1 treated with NaHS. High levels of sFlt1 were induced by an intravenous injection of an adenovirus expressing sFlt1, while healthy control rats received an injection with adenovirus expressing an empty vector. The effect of NaHS on plasma sFlt1, urinary albumin excretion, blood pressure, fetuses, and renal damage was assessed. In addition, we studied the effect of high sFlt1 and H₂S treatment on vascular function in *ex vivo* vascular experiments with thoracic aortic rings.

Animals

All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Committee for Animal Ethical Experiments of the University of Groningen. Female Sprague-Dawley (SD) rats (180-200 g; Charles River, France) were housed in a 12-hour light-dark cycle with constant room temperature. Food and water was provided *ad libitum*. Until selection for experiments, daily vaginal smears were taken. When females were in the pro-oestrus phase of their cycle, they were housed with males overnight. When spermatozoa were present in the vaginal smear the next day, rats were considered to be pregnant (day 0 of gestation).

Safety study

Healthy pregnant rats were treated with intraperitoneal (IP) injections of NaHS, twice daily, from day 12 till day 19 of gestation. The safety-study consisted of three groups; a control group ($n = 4$) that received vehicle (0.9% NaCl), a group receiving 100 $\mu\text{mol/kg}$ NaHS per day ($n = 5$), and a group receiving 400 $\mu\text{mol/kg}$ NaHS per day ($n = 4$). As the IP injections were performed twice daily, the 100 $\mu\text{mol/kg}$ NaHS group received 50 $\mu\text{mol/kg}$ NaHS per IP injection, and the 400 $\mu\text{mol/kg}$ NaHS group received 200 $\mu\text{mol/kg}$ NaHS per IP injection. NaHS was dissolved in 0.9% NaCl.

At day 19 of gestation, rats were anesthetized (isoflurane/oxygen), and prior to termination and harvesting tissues, blood pressure was measured by cannulating the abdominal aorta (bed-side monitor; Datex-Ohmeda, Cardiocap/5). Blood pressure values were noted after 30 seconds of recording. Subsequent to termination of the rats by aortic puncture, uterine horns with fetuses and placentas were removed. The number of implantations, resorptions, and fetuses was counted. The fetal resorption ratio was calculated by dividing the number of resorptions by the number of implantations. Pups and placentas (without mesometrial triangle) were weighted.

Therapeutic effect of NaHS in pregnant rats with high sFlt1

The synthesis of the recombinant adenovirus expressing murine sFlt1(1-3) and control adenovirus (empty vector) was previously described,^{15,16} and kindly provided by Prof. S.A. Karumanchi (Beth Israel Deaconess Medical Center, Boston, MA, USA) and Prof. U.J. Tietge (University Medical Center Groningen, Groningen, the Netherlands). The viruses were amplified in our facility. Briefly, virus was amplified in 293HEK cells. After

24 hours, adenoviral purification was performed with a cesium chloride (CsCl) density gradient. The concentration of plaque forming units (PFU) was analyzed with enzyme-linked immunoassay that detects the adenoviral hexon (Adeasy viral titer kit, Agilent Technologies, Santa Clara, CA, USA), according to the manufacturers' protocol.

Pregnant rats were intravenously injected via the tail vein on day 8 of gestation with 1×10^{12} PFU of adenovirus expressing an empty vector (control rats; $n = 15$) or with the same concentration of adenovirus expressing mouse sFlt1 (sFlt1-rats; $n = 25$). At day 12 of gestation, blood was drawn from the tail vein and plasma mouse sFlt1 levels were determined using a mouse sFlt1 ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA). At the same day, in case the concentration of plasma sFlt1 was increased, treatment with either vehicle (0.9% NaCl) or NaHS (100 $\mu\text{mol/kg}$ in 0.9% NaCl) by daily IP injections for 7 days was started. The NaHS dose of 100 $\mu\text{mol/kg}$ per day was based on its effectivity in previous research,¹³ and was shown to be safe in pregnant rats in our safety experiment. Rats that received virus expressing an empty vector, were all treated with vehicle (control; $n = 15$). Rats that received virus expressing mouse sFlt1 were randomly allocated into either the vehicle treated group (sFlt1 + vehicle; $n = 14$) or the NaHS treated group (sFlt1 + NaHS; $n = 11$).

At day 17 of gestation, rats were housed in metabolic cages for 24 hour to collect urine. At day 19 of gestation, rats were anesthetized (isoflurane/oxygen). Prior to termination and harvesting tissues, blood pressures were measured by cannulating the abdominal aorta (bed-side monitor; Datex-Ohmeda, Cardiocap/5). Blood pressure values were noted after 30 seconds of recording. Blood was collected via the aorta and the right kidney was perfused with 0.9% NaCl and collected. Thereafter, rats were bled. The number of implantations, resorptions, and pups was counted. The fetal resorption ratio was calculated by dividing the number of resorptions by the number of implantations. Length and weight of the pups were measured and fetal brains and livers were collected and weighted to determine brain/liver ratios. Placentas (without mesometrial triangle) were weighted. Finally, the thoracic aorta was isolated and placed in ice-cold, oxygenated Krebs solution. Excess aortic tissue was snap frozen in isopentane on dry ice and stored at -80°C .

Blood and urine analysis

Plasma sFlt1 levels on day 12 and day 19 of gestation were measured using a mouse sFlt1 ELISA kit, according to the manufacturer's protocol (R&D Systems, Inc.). Urinary albumin levels were measured using the Exocell Nephra kit (Philadelphia, PA, USA). In order to

calculate the albumin excretion per 24 hours, the concentration of albumin per sample (mg/mL) was multiplied by the 24-hour urine volume.

Histology and immunohistochemistry

Immediately after harvesting, the right kidney was partly snap frozen in isopentane on dry ice and stored at -80°C. The other part was immediately fixed in paraformaldehyde (4% PFA) for 24 hours, and subsequently stored in 70% ethanol until embedding in paraffin, according to standard methods.

For histological analysis of paraffin embedded renal sections, 2 µm sections were cut and stained with periodic acid–schiff (PAS) and methenamine silver (MS) according to standard methods. Additionally, immunohistochemistry was performed on paraffin embedded renal sections. Total macrophages were identified by staining for CD68 (1:750 diluted; clone ED1; AbD Serotec, Düsseldorf, Germany), as described previously.²⁶ In order to assess renal pre-fibrosis, tissue sections were stained for alpha-smooth muscle actin (α-SMA; 1:10000 diluted; clone 1A4; Sigma), as previously described.²⁷

Immunohistochemistry for RecA, ICAM-1, and CD206 was performed on snap frozen kidney sections. For that purpose, cryostat sections of 4 µm were cut. To identify renal endothelial cells and endothelial cell activation, cryostat sections were stained using a monoclonal antibody against RecA-1, a cell surface antigen expressed by rat endothelial cells (HIS52, 1:5 diluted, derived from hybridoma cells,²⁸) and ICAM-1, a marker for endothelial cell activation (1:5000 diluted; Abcam, Cambridge, UK) respectively. Briefly, cryostat sections were air dried and fixed in acetone, and subsequently incubated with the primary antibody against RecA-1 or ICAM-1 for 1 hour at RT. After blocking endogenous peroxidase with 125 µl 30% H₂O₂ in 50 ml PBS for 30 minutes, incubation with the secondary rabbit anti-mouse and tertiary goat anti-rabbit antibodies (1:50; Agilent Technologies) for 30 minutes at RT was performed. 3-Amino-9-ethylcarbazole (AEC) was used as chromogen. The sections were then counterstained with haematoxylin. Finally, to identify M2-like macrophages in the kidneys, cryostat sections were stained for CD206 (1:1000 diluted; Abcam), as described previously.²⁶

Analysis of kidney sections

Glomerular lesions (PAS staining) and renal ICAM-1 expression were semi-quantitatively scored. The presence of glomerular lesions was divided into four groups; 0 = no glomerular lesions, 1 = mild, 2 = moderate, and 3 = severe glomerular lesions. Renal ICAM-1

(interstitial and glomerular) expression was semi-quantitatively scored for intensity: 1 = low intensity, 2 = moderate intensity, 3 = strong intensity, 4 = very strong intensity. To quantify macrophage influx, interstitial and glomerular ED-1 and CD206 positive cells were manually counted by analyzing 30 randomly selected cortical fields excluding glomeruli (interstitium) or 50 glomeruli (glomerular) per kidney (40x magnification), respectively. Aperio ImageScope v10.2.2.2319 (Vista, CA, USA) was used to quantify the number of α -SMA positive pixels corrected for the area analyzed. Interstitial and glomerular α -SMA positivity was analyzed separately. The *positive pixel Count V9* algorithm was used and vessels were excluded from analysis. Researchers analyzing the kidney sections were blinded for group allocation.

RNA isolation and real-time RT-PCR

RNA was extracted from snap frozen kidney and aortic tissue using the TRIZOL method (Invitrogen, Carlsbad, CA, USA). Total RNA was quantified using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). To synthesize complementary DNA (cDNA), random hexamer primers and Superscript II RT (Invitrogen) were used. Real-time RT-PCR was performed in triplo for angiotensin type 1 receptor (*At1r*) and type 2 receptor (*At2r*) in kidneys and aorta and for *Vegfa* in kidneys. A total volume of 20 μ L containing 10 ng cDNA and 10 μ L PCR-mastermix (Applied Biosystems, Foster City, CA, USA) was used. Primers and probes for *At1r*, *At2r*, and *Vegfa* were obtained from Applied Biosystems (TaqMan Gene Expression Assays, *At1r*: Rn00578456_m1, *At2r*: Rn00560677_m1 and *Vegfa*: Rn01511605_m1). Rat *Hprt* was used as a housekeeping gene, primer forward: 5'-GGCAGTATAATCCAAAGATGGTCAA-3', primer reverse: 5'-GTCTGGCTTATATCCAACACTTCGT-3' (Invitrogen) and probe: 6-FAM 5'-CAAGCTTGCTGGTGAAAAGGACCCC-3' TAMRA (Eurogentec, Liege, Belgium). The thermal profile was 15 min at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The delta Ct was yielded by subtracting the average of the triplo Ct-values for the housekeeping gene *Hprt* from the average of the triplo Ct-values for *At1r*, *At2r*, and *Vegfa*. Results were expressed as $2^{-\Delta Ct}$.

Aortic-ring contraction studies

High sFlt1 is associated with endothelial dysfunction in several diseases.¹⁹⁻²¹ Therefore, the endothelium-dependent relaxation and sensitivity to angiotensin II in aortic tissue was studied by standard isotonic contraction experiments with rat thoracic aorta rings

as previously described.²⁹ Aortic rings (2 mm) were kept in Krebs solution (at 37°C) and gassed with a mixture of 95% CO₂ and 5% O₂. Subsequently, aortic rings were equilibrated for 30 minutes and checked for viability by evoking a contraction with KCl (60 mM) two times for 10 minutes.

Drugs and chemicals

Krebs buffer was freshly made before the start of each experiment and contained: 120 mmol/L sodium chloride (NaCl), 5.9 mmol/L potassium chloride (KCl), 25.2 mmol/L NaHCO₃, 1.2 mmol/L NaH₂PO₄, 10.4 mmol/L glucose, 1.21 mmol/L MgCl₂ · 6H₂O, and 2.52 mmol/L CaCl₂. All ingredients were purchased from E. Merck (Darmstadt, Germany). The stock solutions for phenylephrine (PE; Sigma), acetylcholine (ACh; Sigma), angiotensin II (Bachem AG, Bubendorf, Switzerland), PD-123319 (Pfizer, New York, NY, USA), Losartan (Merck Research Laboratories, Rahway, NJ, USA), and NG-nitro-L-arginine methyl ester (L-NMMA; Calbiochem, San Diego, CA, USA) were prepared in 0.9% NaCl. Indomethacin (Sigma) was dissolved in NaHCO₃.

Ex vivo endothelium-dependent relaxation

The total endothelium-dependent relaxation of the aortic rings was tested in duplo by obtaining a cumulative ACh concentration-response curve (10⁻⁸ M - 10⁻⁴ M) after pre-contraction of the rings with 10⁻⁶ M phenylephrine. Subsequently, the NO donor sodium nitroprusside (SNP, 10⁻⁵ M, Sigma) was added as a control for endothelium-independent relaxation. To assess the contribution of NO, prostaglandins and endothelial derived hyperpolarization factor (EDHF) to the total endothelium-dependent relaxation, ACh mediated relaxation was performed in aortic rings in the presence of respectively the selective nitric oxide (NO) synthase inhibitor L-NMMA, the cyclooxygenase (COX) inhibitor indomethacin or both. The mean ACh-mediated relaxation of the duplo rings in each condition was calculated as a percentage of the phenylephrine mediated pre-contraction.

Ex vivo aortic reactivity to angiotensin II

The angiotensin II mediated contraction of the aortic rings was tested in duplo by obtaining a cumulative angiotensin II concentration-response curve (10⁻¹⁰ M - 10⁻⁶ M). The angiotensin II mediated contraction via solely the AT1-R was studied by obtaining the same angiotensin II concentration-response curve (10⁻¹⁰ M - 10⁻⁶ M), but in the presence

of 10^{-6} M PD-123319 (AT2-R antagonist) and L-NMMA (10^{-4} M). Moreover, the angiotensin II mediated response via the AT2-R was determined by obtaining the angiotensin II concentration-response curve (10^{-10} M - 10^{-6} M) in the presence of 10^{-5} M Losartan (AT1-R antagonist). Angiotensin II mediated contraction was calculated as a percentage of total contraction, which was determined after each experiment by adding 10^{-5} M phenylephrine to the aortic rings. Angiotensin II mediated relaxation (via the AT2-R) was calculated as a percentage of pre-contraction induced by 10^{-5} M phenylephrine.

Statistics

Data were analyzed using SPSS 22.0 (SPSS Inc. Chicago, IL, USA) and GraphPad prism 5.01 (GraphPad Software Inc. San Diego, CA, USA). Parametric data are presented as mean \pm standard error of the mean (SEM) and non-parametric data as median (interquartile range). Unless otherwise stated, differences between the three groups were tested using One-Way ANOVA followed by post-hoc LSD analysis or Kruskal-Wallis followed by Dunn's post-hoc analysis for parametric or non-parametric data, respectively. For correlation of parameters, Spearman correlation coefficients were generated. Outliers were assessed using the formula 1.5 times the interquartile range. A p -value < 0.05 was considered to be statistically significant.

Results

Safety study

We first tested the safety of NaHS treatment in healthy pregnant rats. The characteristics of the rats are summarized in table 1. No effect of NaHS was observed on maternal weight at day 19 of gestation, maternal mean arterial blood pressure (MAP), litter size or fetal resorption ratio. No macroscopic anomalies of the pups were observed after NaHS treatment. The weight of the pups at day 19 of gestation was higher in both NaHS treated groups compared with the control group. These differences were not accompanied by an increase in placental weight. The placental/fetal weight ratio was significantly lower in the rats treated with 200 μ M NaHS as compared to controls (table 1).

Table 1 - Safety of NaHS treatment in pregnant rats

	Control (n = 4)	100 uM NaHS (n = 5)	400 uM NaHS (n = 4)
Maternal weight (grams)	351 ± 10	327 ± 10	321 ± 11
Maternal MAP (mmHg)	98 ± 7	103 ± 4	104 ± 7
Litter size (n)	14 ± 1	11 ± 2	11 ± 0
Fetal resorption ratio	0.10 (0.03 – 0.10)	0.10 (0.00 – 0.20)	0.15 (0.03 – 0.20)
Fetal weight (grams)	1.368 (1.270 – 1.444)	1.436 (1.354 – 1.513)*	1.482 (1.356 – 1.576)*
Placental weight (grams)	0.374 (0.354 – 0.403)	0.350 (0.325 – 0.390)	0.377 (0.337 – 0.421)
Placental/fetal weight ratio	0.279 (0.257 – 0.297)	0.252 (0.230 – 0.273)*	0.256 (0.234 – 0.292)

Characteristics of vehicle treated pregnant rats (control), pregnant rats treated with 100 uM NaHS daily (100 uM NaHS), and pregnant rats treated with 400 uM NaHS daily (400 uM NaHS) are shown. MAP; mean arterial pressure, fetal resorption ratio; number of resorptions divided by number of implantations. Data are presented as mean ± SEM or median (interquartile range) and analyzed using the one-Way ANOVA followed by post-hoc LSD analysis or the Kruskal Wallis followed by Dunn's post-hoc analysis, **p*-value < 0.05 versus control.

Therapeutic effect of NaHS in pregnant rats with high sFlt1

Pregnant rats with sFlt1 overexpression did not develop hypertension or albuminuria.

Since we found no negative effects of NaHS on the mother or the fetuses, the potential therapeutic effects of NaHS were evaluated in pregnant rats with high plasma sFlt1 in our second experiment. In this experiment, three groups were included; healthy pregnant rats that received adenovirus with an empty vector and vehicle treatment (controls), pregnant rats that received sFlt1-adenovirus that were treated with vehicle (sFlt1 + vehicle), and pregnant rats that received sFlt1-adenovirus that were treated with NaHS (sFlt1 + NaHS). Infection with mouse sFlt1-adenovirus resulted in a wide range of plasma mouse sFlt1 levels (table 2). Median plasma sFlt1 levels at day 12 and 19 were significantly higher in both the sFlt1 + vehicle and sFlt1 + NaHS groups as compared to controls. Plasma sFlt1 levels were similar between sFlt1 + vehicle and sFlt1 + NaHS groups and between day 12 and day 19 of gestation for all groups (table 2).

Table 2 - Plasma mouse sFlt1 on day 12 and day 19 of gestation

Plasma mouse sFlt1 (ng/mL)	Control (n = 14) ¹	sFlt1 + vehicle (n = 14)	sFlt1 + NaHS (n = 11)
Gestational age day 12	0.09 (0.06 – 0.10)	175 (47 – 670)*	379 (38 – 1033)*
Gestational age day 19	0.08 (0.06 – 0.09)	134 (16 – 207)*	561 (19 – 1246)*

Median (interquartile range) plasma mouse sFlt1 of healthy pregnant rats (control), pregnant sFlt1-rats treated with vehicle (sFlt1 + vehicle), and pregnant sFlt1-rats treated with 100 μ M NaHS daily (sFlt1 + NaHS) are shown. Data were analysed using the Kruskal Wallis followed by Dunn's post-hoc analysis, * p -value < 0.05 versus control. ¹Plasma free sFlt1 in control pregnant rats was determined in $n = 4$.

No significant differences in MAP were observed between the three groups (figure 1A). Moreover, no differences were found in the amount of albumin excreted in urine per 24 hours between the three groups (figure 1B). Because of the wide range of sFlt1 levels, we wondered whether there was a correlation between the circulating sFlt1 levels and either blood pressure or albuminuria. For both sFlt1 + vehicle and sFlt1 + NaHS rats, no significant correlations were found between plasma sFlt1 on day 19 of gestation and mean arterial blood pressure ($r = -0.636$, $p = 0.175$ and $r = -0.188$, $p = 0.628$ respectively). We also did not find significant correlations between plasma sFlt1 on day 19 of gestation and albumin excretion per 24 hours for sFlt1 + vehicle ($r = 0.095$, $p = 0.748$) and sFlt1 + NaHS rats ($r = -0.018$, $p = 0.960$).

NaHS restored the brain/liver ratio in pups from mothers with high sFlt1 during pregnancy

Fetal data are summarized in table 3. No differences were observed between the groups with regard to litter size or resorption ratio. There were no differences observed in fetal weight and length between the three groups as well. However, as compared to controls, pups from sFlt1-rats that were treated with vehicle showed a decreased brain/liver ratio. Pups from sFlt1-rats treated with NaHS showed a higher brain/liver ratio as compared to the vehicle treated sFlt1 group. The weight of the placentas from sFlt1 + vehicle and sFlt1 + NaHS rats was higher as compared to controls. In comparison to the controls, the fetal/placental weight ratio was increased in the sFlt1 + vehicle and sFlt1 + NaHS rats (table 3).

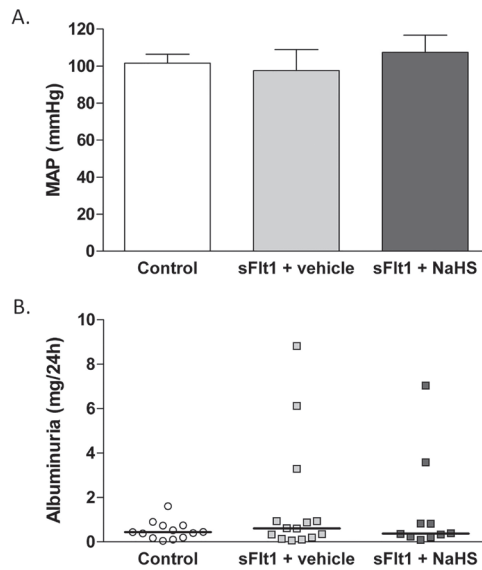


Figure 1 - Maternal blood pressure and albuminuria

Mean arterial pressure (MAP, mmHg) (A) and albuminuria (mg/24h) (B) of healthy pregnant rats (control), pregnant sFlt1-rats with vehicle treatment (sFlt1 + vehicle), and pregnant sFlt1-rats with NaHS treatment (sFlt1 + NaHS) are shown. Blood pressure data (A) is shown as mean (+ standard deviation), and analysed using the one-Way ANOVA followed by post-hoc LSD analysis. Medians for albuminuria are indicated (B), differences in albumin excretion were tested using the Kruskal Wallis followed by Dunn's post-hoc analysis. No differences were observed.

Table 3 - Fetal characteristics after maternal sFlt1 overexpression and NaHS treatment

	Control (n = 14)	sFlt1 + vehicle (n = 15)	sFlt1 + NaHS (n = 11)
Litter size (n)	14 ± 0.8	13 ± 1.0	14 ± 0.9
Fetal resorption ratio	0.00 (0.00 – 0.00)	0.00 (0.00 – 0.02)	0.00 (0.00 – 0.06)
Fetal weight (grams)	2.267 ± 0.011	2.230 ± 0.018	2.306 ± 0.025
Fetal length (mm)	27.82 ± 0.102	27.61 ± 0.174	27.86 ± 0.197
Brain/liver ratio	0.590 ± 0.008	0.538 ± 0.008*	0.611 ± 0.161 [‡]
Placental weight (grams)	0.402 ± 0.003	0.442 ± 0.006*	0.428 ± 0.013*
Placental/fetal weight ratio	0.178 ± 0.002	0.191 ± 0.003*	0.189 ± 0.003*

Characteristics of the pups from healthy pregnant rats (control), pregnant sFlt1-rats treated with vehicle (sFlt1 + vehicle), and pregnant sFlt1-rats treated with NaHS (sFlt1 + NaHS) are shown. Resorption ratio; number of resorptions divided by number of implantations. Data are presented as mean ± SEM or median (interquartile range) and analyzed using the one-Way ANOVA followed by post-hoc LSD analysis or Kruskal Wallis followed by Dunn's post-hoc analysis, **p*-value < 0.05 versus control, [‡]*p*-value < 0.05 versus sFlt1.

NaHS did not affect high sFlt1 induced glomerular lesions

It has been shown previously that high sFlt1 levels in pregnant rats were associated with glomerular endotheliosis.¹⁹ In our model, glomerular lesions were seen in sFlt1-rats with no effect of NaHS; the lesions were not observed in control rats (figure 2D). As shown in figure 2B and 2C, the glomerular lesions are characterized by an increase in glomerular PAS-positive material and narrowing of the lumen of glomerular capillaries. The latter might be due to swollen endothelial cells or mesangial accumulation of extracellular matrix proteins.

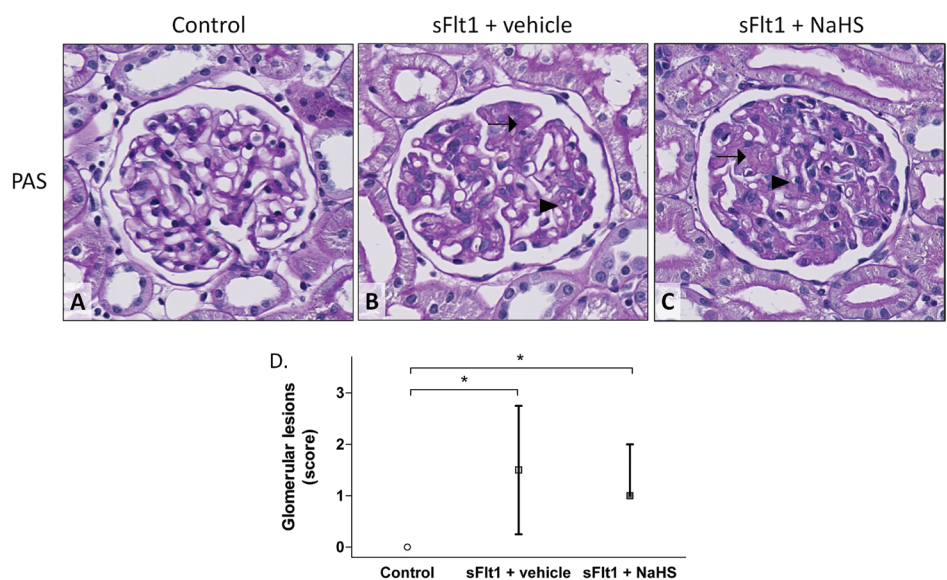


Figure 2 - Glomerular lesions

Representative photomicrographs of PAS stained renal sections from healthy pregnant rats (control) (A), pregnant sFlt1-rats receiving vehicle treatment (B), and pregnant sFlt1-rats receiving NaHS (C). Increased glomerular PAS-positive lesions (arrows) with narrowing of the glomerular capillaries (arrow-heads) is seen in sFlt1-rats that received vehicle (B) and NaHS (C). Semi-quantitative analyses of glomerular lesions was shown in (D), medians and interquartile range are indicated. Data were analyzed using the Kruskal Wallis followed by Dunn's post-hoc analysis, *p-value < 0.05. Original magnification, x40.

To characterize the glomerular lesions in more detail, additional immunohistochemical stainings were performed. Since we observed no effect of NaHS on these lesions, we only used sections of control rats and sFlt1 + vehicle rats. To study endothelial cells in more detail, we stained the sections for the endothelial cell marker RecA-1. This staining revealed an increase in RecA-1 positivity (figure 3B) in sFlt1 rats, as compared to controls. To identify changes in the glomerular basal membrane (GBM), a methenamine silver (MS) staining was performed. As shown in figure 3D, thickening and segmental duplication of the GBM can be observed in sFlt1 overexpressing rats. Furthermore, the MS staining also suggests increased mesangial matrix expansion (figure 3D). Unfortunately, electron microscopy could not be performed to confirm glomerular endotheliosis.

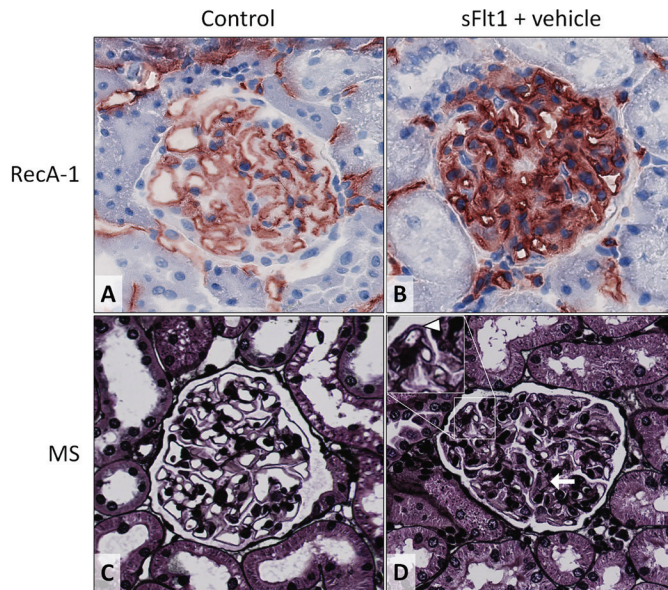


Figure 3 - Glomerular RecA-1 expression and methenamine silver staining

RecA-1 stained sections from healthy pregnant rats (control) (A), and pregnant sFlt1-rats receiving vehicle treatment (sFlt1 + vehicle) (B). MS stained sections from healthy pregnant rats (control) (C), and pregnant sFlt1-rats receiving vehicle treatment (sFlt1 + vehicle) (D). Changes of the basal membrane with mesangial matrix expansion (white arrow) and thickening / segmental duplication of the glomerular basal membrane (white arrow-head) are revealed in sFlt1-rats, but not in controls. Original magnification, x40.

NaHS did not affect high sFlt1 induced renal inflammation and profibrotic injury

To evaluate whether the glomerular lesions found in this model are associated with an increased inflammation, the influx of total macrophages (ED1-positive) and M2-macrophages (CD206-positive) were analyzed. The sFlt1 + vehicle rats show a significantly increased number of ED-1 positive cells in both glomeruli and interstitium as compared to the controls. There was no effect of NaHS on numbers of ED-1 cells (figure 4A and 4B). Representative micrographs of the ED-1 staining are shown in figure 4F, 4G, 4H for all groups. While no differences were observed in the number of CD206 positive cells in the interstitium between groups (figure 4D), CD206 positive cells were significantly upregulated in the glomeruli of sFlt1 + vehicle and sFlt1 + NaHS rats as compared to controls (figure 4C). As endothelial ICAM-1 expression is important in the migration of monocytes from the vessels to the tissues, we evaluated whether endothelial ICAM-1 expression was upregulated in sFlt1 rats. Indeed, a significant increase in ICAM-1 expression was observed in the sFlt1 + vehicle rats as compared with control rats. In sFlt1 rats treated with NaHS, a trend towards an increased ICAM-1 expression was observed (figure 4E). ICAM-1 expression significantly correlated with sFlt1 plasma levels ($r = 0.548$, $p = 0.004$), ED1 and CD206 positive cells in the glomeruli (respectively $r = 0.702$, $p < 0.001$, and $r = 0.692$, $p < 0.001$). No correlation between ICAM-1 expression and interstitial ED1 and CD206 positive cells was observed (respectively $r = -0.011$, $p = 0.961$, and $r = -0.453$, $p = 0.162$).

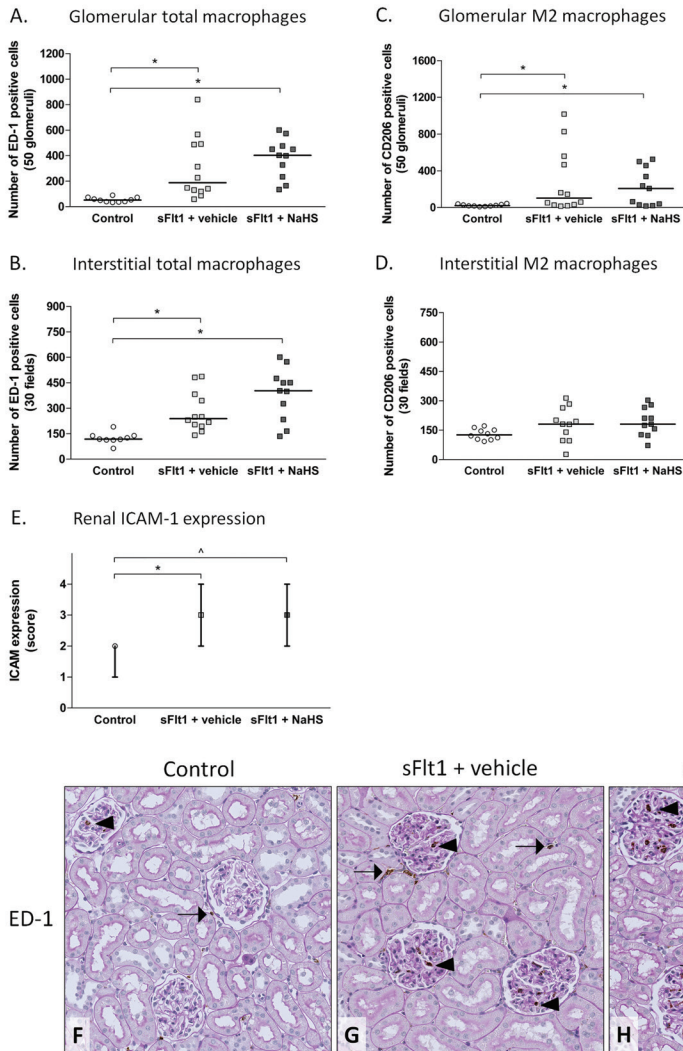


Figure 4 - Renal macrophage influx and ICAM-1 expression

The number of ED-1 positive cells in the glomeruli (A) and interstitium (B), and the number of CD206-positive cells in the glomeruli (C) and interstitium (D), and ICAM expression (interstitium + glomeruli) (E) in the kidneys of healthy pregnant rats (control), pregnant sFlt1-rats receiving vehicle treatment (sFlt1 + vehicle), and in pregnant sFlt1-rats receiving NaHS (sFlt1 + NaHS) are shown. For A-D, medians are indicated by bars. For ICAM expression (E), medians and interquartile range are given. ED-1 positive cells are present in both renal interstitium (arrows), and glomeruli (arrow-heads). Representative micrographs of the ED-1 staining are shown for controls (F), sFlt1 + vehicle (G) and sFlt1 + NaHS (H) rats. Data were analyzed using the Kruskal Wallis followed by Dunn's post-hoc analysis, * p -value < 0.05, ^ p -value < 0.1 (borderline significant). Original magnification, x20.

As shown in figure 5A and 5B, the number of α SMA-positive pixels was significantly increased in both renal interstitium and glomeruli of sFlt1 + vehicle rats as compared to the control rats, with no effect of NaHS treatment (figure 5A and 5B). Although significantly increased in sFlt1 treated rats, the increase of renal α SMA-positivity is mild in both sFlt1 + vehicle and sFlt1 + NaHS groups (figure 5C, 5D, 5E).

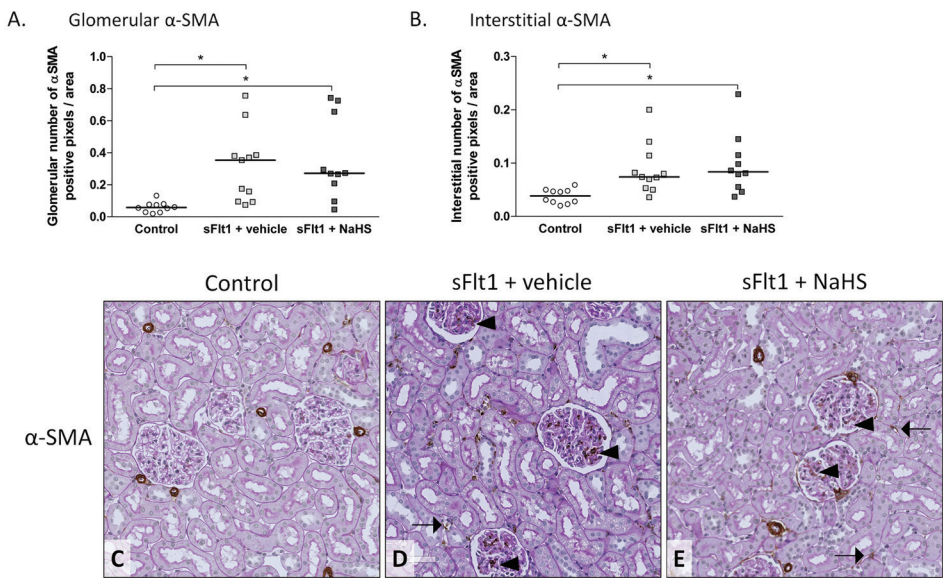


Figure 5 - Prefibrotic lesions in the kidney

The number of α SMA positive pixels in the glomeruli (A) and interstitium (B) in the kidney of healthy pregnant rats (control), pregnant sFlt1-rats receiving vehicle treatment (sFlt1 + vehicle), and in pregnant sFlt1-rats receiving NaHS (sFlt1 + NaHS) are shown. Medians are indicated. Representative micrographs of the α SMA staining is shown for controls (C), sFlt1 + vehicle (D), and sFlt1 + NaHS (E) rats. α SMA positivity is present in both renal interstitium (arrows) and glomeruli (arrow-heads). Data were analyzed using the Kruskal Wallis followed by Dunn's post-hoc analysis, * p -value < 0.05. Original magnification, x20.

NaHS treatment has no effects on renal gene expression of Vegfa

Within the kidney, VEGF-A is constitutively produced by podocytes and maintains endothelial health within the glomerulus. It has been shown that glomerular VEGF-A deficiency caused glomerular endotheliosis by loss of fenestrated endothelium.³⁰ NaHS can increase renal *Vegfa* mRNA expression.¹³ We therefore studied whether the renal production of *Vegfa* mRNA is aberrant in the present rat model with high sFlt1 and whether

NaHS treatment increased *Vegfa* gene expression. As shown in figure 6, no differences in *Vegfa* mRNA expression between the three groups were observed.

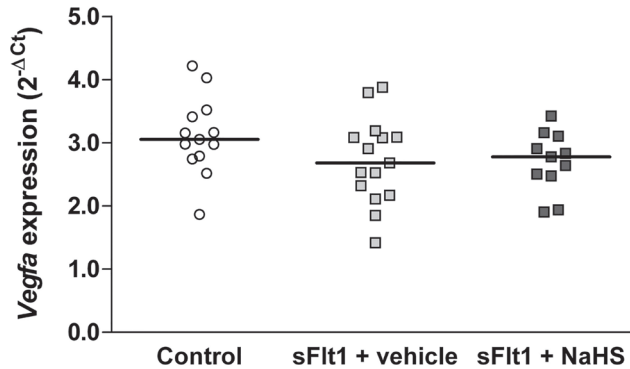


Figure 6 - *Vegfa* mRNA expression in the kidney

Vegfa mRNA ($2^{-\Delta C_t}$) expression in the kidney of healthy pregnant rats (control), pregnant sFlt1-rats receiving vehicle (sFlt1 + vehicle) and pregnant sFlt1-rats receiving NaHS (sFlt1 + NaHS) are shown. Medians are indicated. Data were analyzed using the Kruskal Wallis followed by Dunn's post-hoc analysis, * p -value < 0.05.

NaHS did not affect total acetylcholine-mediated relaxation, nor the role of NO, prostaglandins and EDHF in relaxation

High sFlt1 levels are thought to contribute to the development of vascular dysfunction.^{21,31} Therefore, we studied *ex vivo* endothelial function. Aortic rings were pre-contracted with phenylephrine and dilated with acetylcholine (ACh). There were no differences observed in the total ACh-mediated relaxation curves between the three groups (figure 7A), neither in the $\log EC_{50}$ and E_{max} of the total ACh-mediated relaxation (table 4). To identify the contribution of NO, prostaglandins or remaining factors such as EDHF to the total ACh-mediated relaxation, the aortic rings were incubated with respectively L-NMMA, indomethacin or both. The percentage of contribution to the total ACh-mediated relaxation of NO, PG, and EDHF is depicted in figure 7B-7D. There were no differences observed between the three groups. Furthermore, there were no correlations found between percent contribution of NO, PG and EDHF with sFlt1 plasma levels (data not shown). As shown in table 4, no differences between groups were observed for the $\log EC_{50}$ and E_{max} of the ACh-mediated response after incubation with either vehicle, L-NMMA, indomethacin or both.

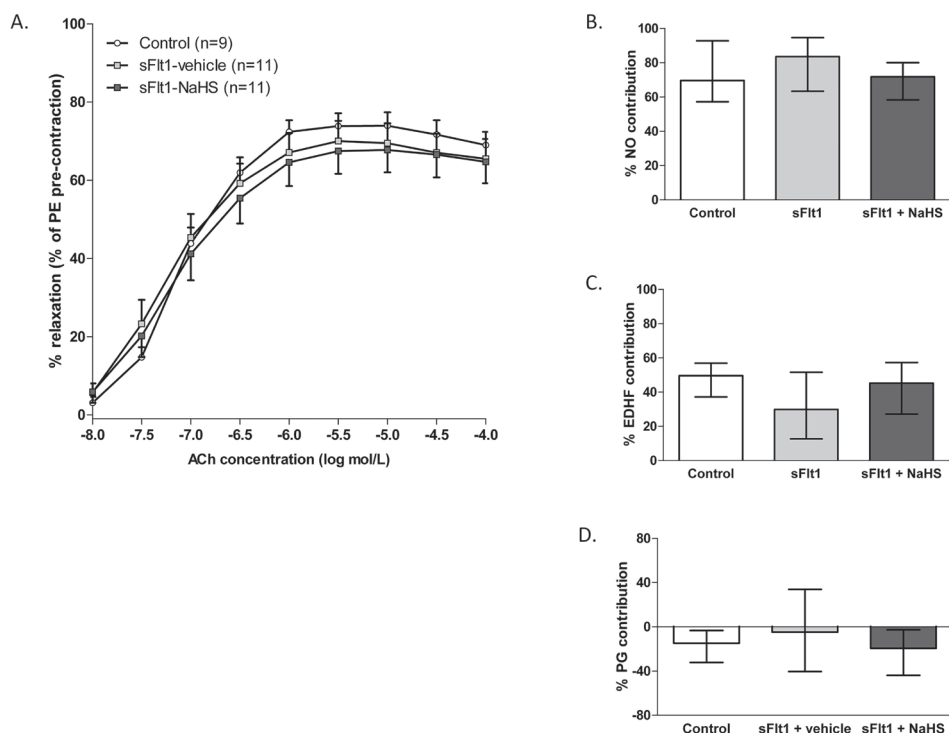


Figure 7 - Cumulative acetylcholine dose-response curves

(A) The mean \pm SEM of the cumulative acetylcholine (ACh) dose-response curves in the thoracic aorta from healthy pregnant rats (control), pregnant sFlt1-rats treated with vehicle (sFlt1 + vehicle), and pregnant sFlt1-rats treated with NaHS (sFlt1 + NaHS) is shown. The median (interquartile range) percentage of nitric oxide (NO) (B), prostaglandins (PG) (C) and endothelial derived hyperpolarization factor (EDHF) (D) contribution to the total ACh-mediated relaxation in healthy pregnant (control), sFlt1-rats with vehicle treatment (sFlt1 + vehicle), and sFlt1-rats with NaHS treatment (sFlt1 + NaHS) are shown. Data were analyzed using Repeated Measures ANOVA (A) and One-Way ANOVA followed by post-hoc LSD analysis (B, C, and D), no significant differences were observed. PE; phenylephrine.

NaHS treatment attenuates sensitivity to angiotensin II in sFlt1-rats

To study angiotensin II sensitivity, aortic rings were incubated with increasing doses of angiotensin II and response was measured. The sensitivity to angiotensin II mediated contraction was increased in pregnant sFlt1-rats receiving vehicle treatment as compared to both control ($p = 0.051$) and sFlt1-rats receiving NaHS treatment ($p = 0.064$) (figure 8A). The E_{max} of angiotensin II mediated contraction was significantly lower in the sFlt1-rats

treated with NaHS compared to the sFtl1 + vehicle group (table 5). Although angiotensin II mediated contraction seemed to be increased in sFtl1+vehicle compared with the other 2 groups, after inhibition of the AT2-R with PD123319 (and NO with L-NMMA) there were no significant differences between the three groups (figure 8B). Blocking the AT1-R with Losartan also resulted in angiotensin II-mediated contraction, which is in contrast to what was expected.³² There were no significant differences between the three groups after blocking the AT1-R with Losartan (figure 8C). No differences were observed in the $\log EC_{50}$ and E_{max} of angiotensin II mediated contraction between the three groups after blocking the AT2-R and NO synthase with respectively PD123319 and L-NMMA, or the AT1-R with Losartan (table 5).

NaHS treatment increases At2r mRNA expression in the kidney

To determine whether altered angiotensin II sensitivity is related to differential expression of its receptors, *At1r* and *At2r*, mRNA expression levels of these receptors were assessed in the aorta (figure 9A and 9B) and kidney (figure 9C and 9D). No difference in aortic *At1r*, *At2r* mRNA expression was observed between the groups. No differences were observed for renal *At1r* mRNA expression between the three groups. However, *At2r* mRNA in the kidney was significantly increased in sFtl1 rats treated with NaHS, as compared to sFtl1-rats treated with vehicle.

Table 4 - $\log EC_{50}$ and E_{max} of the endothelial function dose response curves

		Endothelial function		
		Control (n = 9)	sFtl1 + vehicle (n = 10)	sFtl1 + NaHS (n = 10)
Vehicle	$\log EC_{50}$	-7.35 ± 0.11	-7.46 ± 0.45	-7.23 ± 0.40
	E_{max}	74.09 ± 9.5	68.99 ± 16.46	67.69 ± 18.68
L-NMMA	$\log EC_{50}$	-7.20 ± 0.24	-6.99 ± 0.44	-7.07 ± 0.39
	E_{max}	21.50 ± 15.35	25.20 ± 21.29	24.11 ± 14.17
Indometacin	$\log EC_{50}$	-7.42 ± 0.15	-7.28 ± 0.56	-7.18 ± 0.36
	E_{max}	82.87 ± 9.87	70.83 ± 21.20	82.67 ± 7.23
L-NMMA + Indometacin	$\log EC_{50}$	-7.07 ± 0.27	-6.98 ± 0.39	-7.09 ± 0.32
	E_{max}	37.56 ± 16.02	31.67 ± 20.71	33.48 ± 18.72

Mean \pm SEM of $\log EC_{50}$ and E_{max} of the endothelial function dose response curves of healthy pregnant rats (control), pregnant sFtl1-rats treated with vehicle (sFtl1 + vehicle), and pregnant sFtl1-rats treated with NaHS (sFtl1 + NaHS) are shown. L-NMMA: NG-nitro-L-arginine methyl ester. Data were analyzed using the one-Way ANOVA followed by post-hoc LSD analysis, no differences between groups were observed.

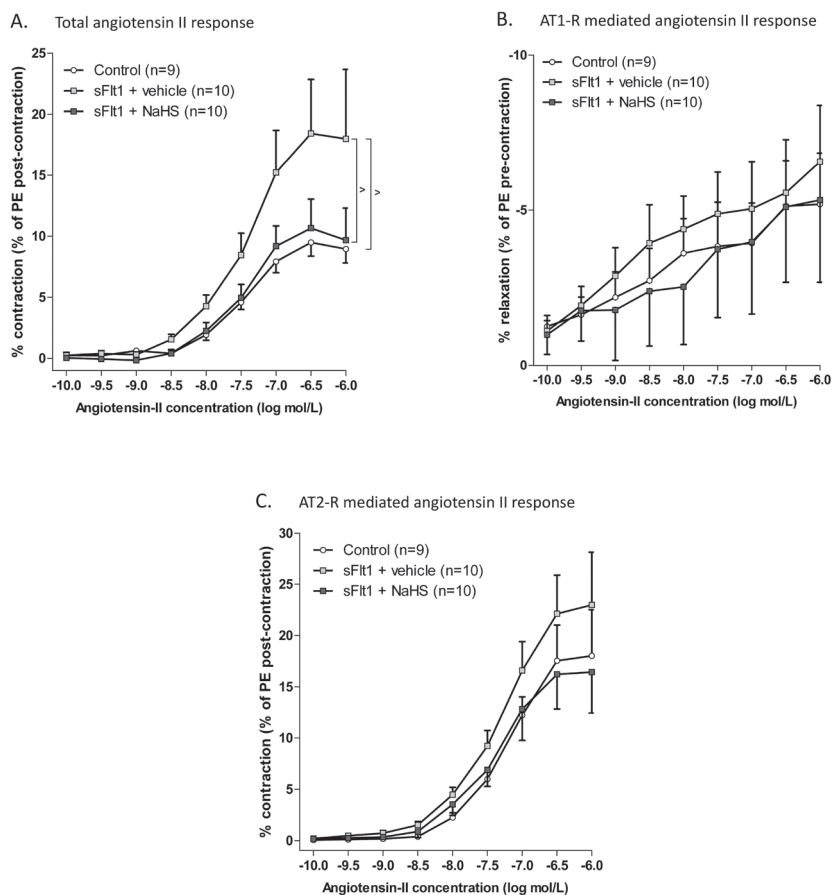


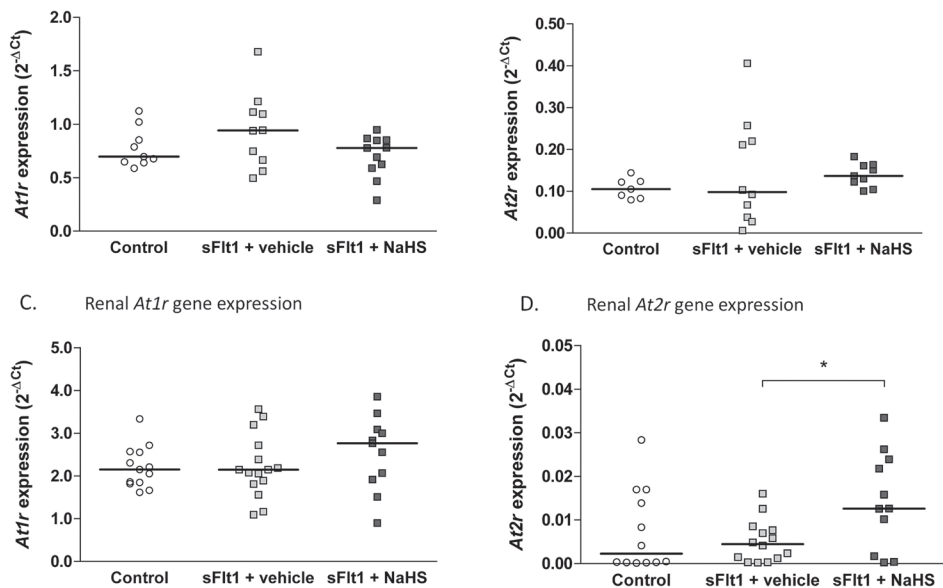
Figure 8 - Cumulative angiotensin II dose-response curves

The mean \pm SEM of the cumulative angiotensin II dose-response curves in the thoracic aorta from healthy pregnant rats (control), pregnant sFlt1-rats with vehicle treatment (sFlt1 + vehicle), and sFlt1-rats with NaHS treatment (sFlt1 + NaHS). (A) Contraction induced by angiotensin II without the presence of inhibitors; (B) Contraction induced by angiotensin II in the presence of the AT2-R antagonist PD123319 and L-NMMA; (C) Contraction induced by angiotensin II in the presence of the AT1-R antagonist Losartan. Data were analyzed using Repeated Measures ANOVA. $^{\wedge}p$ -value < 0.1 versus controls (borderline significant). PE, phenylephrine.

Table 5 - Log EC₅₀ and E_{max} of angiotensin II sensitivity dose response curves

		Angiotensin II sensitivity		
		Control (n = 9)	sFlt1 + vehicle (n = 10)	sFlt1 + NaHS (n = 10)
Angiotensin II	Log EC ₅₀	-7.48 ± 0.18	-7.58 ± 0.38	-7.47 ± 0.24
	E _{max}	10.69 ± 3.92	15.29 ± 9.91	8.80 ± 3.34*
AT1-R response	Log EC ₅₀	-7.31 ± 0.31	-7.34 ± 0.35	-7.57 ± 0.26
	E _{max}	13.17 ± 3.23	26.24 ± 17.3	24.06 ± 20.16
AT2-R response	Log EC ₅₀	-8.31 ± 0.81	-8.13 ± 0.95	-8.22 ± 0.81
	E _{max}	-7.12 ± 0.59	-6.32 ± 4.85	-5.04 ± 8.44

Mean ± SEM of log EC₅₀ and E_{max} of the angiotensin II sensitivity dose response curves of healthy pregnant rats (control), pregnant sFlt1-rats treated with vehicle (sFlt1 + vehicle), and pregnant sFlt1-rats treated with NaHS (sFlt1 + NaHS) are shown. Data were analyzed using the one-Way ANOVA followed by post-hoc LSD analysis, **p*-value < 0.05.

Figure 9 - *At1r* and *at2r* mRNA expression in the kidney and aorta

The angiotensin receptor-1 (*At1r*) mRNA (2^{-ΔCt}), and angiotensin receptor (*At2r*) mRNA (2^{-ΔCt}) in the aorta (A, B) and kidney (D, E) of healthy pregnant rats (control), pregnant sFlt1-rats receiving vehicle (sFlt1 + vehicle) and pregnant sFlt1 rats receiving NaHS (sFlt1 + NaHS) are shown. Medians are indicated. Differences in mRNA expression of *At1r* and *At2r* between sFlt1-rats receiving vehicle and sFlt1-rats receiving NaHS were tested by using the Mann Whitney U test, **p*-value < 0.05.

Discussion

The present study was designed to evaluate the therapeutic effect of H₂S in pregnant rats with high plasma levels of sFlt1. High sFlt1 levels in pregnant rats have been described to induce preeclampsia-like signs.¹⁹ In the current setting, overexpression of sFlt1 did not induce hypertension or albuminuria in the pregnant rats. Due to the lack of hypertension and proteinuria, we were not able to answer our main study question whether NaHS might have therapeutic potential in experimental preeclampsia.

In our experimental setting, several renal lesions were observed in rats with high plasma levels of sFlt1. In the glomeruli in the kidneys of these rats, an increase in PAS-positive material, thickening of the basal membrane, and narrowed capillary lumens were observed. Furthermore, increased renal influx of macrophages, and prefibrotic changes in the kidney of pregnant rats with high sFlt1 were observed. The present study also showed that high levels of sFlt1 in the pregnant rats affected vascular function by increasing angiotensin II sensitivity in the aorta. NaHS treatment slightly affects fetal brain to liver ratio in high sFlt1 pregnant rats, but did not affect glomerular lesion in these rats. This latter lack of effect may be due to the lack of increase of VEGF production in the kidney in these animals. However, we observed that angiotensin II sensitivity in sFlt1 rats was decreased by NaHS treatment.

The main objective of the present study was to investigate the effect of NaHS in pregnant rats with high sFlt1, since high levels of sFlt1 were known to induce a preeclampsia-like syndrome characterized by hypertension and proteinuria.^{13,19,37,38} However, in contrast to the other studies^{13,19,37,38}, overexpression of sFlt1 in our study did not induce hypertension or proteinuria in pregnant rats. The reason for this lack of hypertension and proteinuria in the present study is unknown. The median level of sFlt1 in the current study is comparable with the average levels in the study of Maynard et al.,¹⁹ who found hypertension and proteinuria in pregnant rats with high sFlt1 induced by the same adenovirus. Because the levels of sFlt1 in the present study were variable, ranging from 6.5 to 20,000 ng/mL, we correlated sFlt1 plasma levels with blood pressure and albumin excretion. However, no correlations with sFlt1 plasma levels were found. Variability in sFlt1 levels can therefore not explain the lack of hypertension and albuminuria in our study. Apparently, high sFlt1 levels do not necessarily induce a preeclampsia-like phenotype. In the present experimental setting, induction of high sFlt1 levels in pregnant rats is not a suitable model for preeclampsia. However, in our opinion, the model is still suitable for evaluating tissue effects of high circulating levels of free sFlt1 during pregnancy.

Indeed, glomerular lesions were observed in the rats overexpressing sFlt1. These consisted of extracellular matrix expansion and/or swollen endothelium, resulting in narrowing of the capillary lumen. Furthermore, segmental duplication and thickening of the GBM were observed in the MS staining, these lesions are characteristic for glomerular endotheliosis.³⁹ Whether glomerular endotheliosis is present in our model should be assessed by electron microscopy. Interestingly, the glomerular lesions in the current study were present in absence of hypertension or proteinuria. This is in agreement with studies that observed glomerular endotheliosis in pregnant women without hypertension or proteinuria,^{40,41} and with the hypothesis that VEGF deprivation is the inducer of glomerular endotheliosis in women with PE.⁴² Within the kidney, VEGF is constitutively produced by podocytes and serves an important factor for the maintenance of glomerular health and induction of endothelial fenestrae.³⁰ Apparently, the mechanisms of sFlt1 to induce hypertension and proteinuria are different from those inducing local effects in the kidney, underlying mechanism for this differential effect is unknown.

Although rats with high levels of sFlt1 did not develop albuminuria in the present study, mild prefibrotic changes in both interstitium and glomeruli were observed. The renal α -SMA positivity in rats with high sFlt1 (receiving vehicle or NaHS treatment) correlated with the albumin excretion per 24 hour ($r = 0.797$, $p < 0.001$ for interstitial α -SMA positivity, and $r = 0.682$, $p < 0.001$ for glomerular α -SMA positivity; data not shown). We therefore hypothesize that the time from the induction of high sFlt1 to sacrifice of the rats (i.e. 7-8 days) might have been too short to induce more prefibrotic changes and subsequent albuminuria. Others described interstitial and glomerular fibrosis in a rat model with experimental kidney disease and sFlt1 overexpression after 56 days.⁴⁶

H₂S is a gaseous signalling molecule, which is known for its vasodilatory, cytoprotective and pro-angiogenic properties, among others.³³ An important target of H₂S is the endothelium, where the gas contributes to maintaining endothelial function.² Our group recently showed NaHS (as an H₂S donor) has beneficial effects in a rat model with generalized endothelial dysfunction, induced by high free plasma sFlt1 levels.¹³ In this previous study, NaHS treatment attenuated hypertension, proteinuria, and glomerular endotheliosis. Moreover, NaHS shifted the anti-angiogenic state towards pro-angiogenic, by upregulation of free plasma VEGF.¹³ Because the effects of H₂S treatment have never been investigated in pregnant rats before, we first performed a safety-study using two different concentrations of NaHS (100 μ mol/kg and 400 μ mol/kg NaHS per day). We revealed no harmful effects of both NaHS concentrations on as well maternal

as fetal parameters. Markedly, in the safety-study, treatment with both concentrations of NaHS resulted in increased fetal weights, without any changes in placental weight. This might indicate an increased blood flow towards the fetoplacental vasculature, and thus increased supply of oxygen and nutrients to the fetus. Indeed, it was shown by others that H_2S is able to increase blood flow and organ perfusion.⁸ Whether H_2S is also able to increase blood flow in the fetoplacental vasculature should be examined in the future, for example by ultrasound Doppler techniques. Moreover, NaHS could have had direct effects on the fetuses. For example via up regulation of insulin-like growth factor (IGF) which is associated with fetal growth regulation.^{12,13}

Since we found no major effect of NaHS on mother and fetuses in our safety study, we studied the effect of NaHS in pregnant rats with high sFlt1. Various effects of H_2S were observed in fetuses of pregnant sFlt1-rats. Although no effects on fetal weight and length were observed, we observed a lower brain/liver ratio in sFlt1 rats treated with vehicle. This indicates a decreased brain growth versus liver growth, and may suggest decreased placental function. This suggestion is in line with the fact that placentas of sFlt1 overexpressing rats have increased placental weight, which may be a compensatory mechanism for placental insufficiency. NaHS seemed to reverse the fetal brain/liver ratio, suggesting that NaHS does increase placental function. This suggestion is in accordance with our safety-study, which also suggested increased placental function after treatment with H_2S . Taken together, the current data suggests that H_2S has beneficial effects on fetal growth, possibly by enhancing blood flow.

We found no effect of NaHS treatment on the glomerular lesions in the present study. Previously, however, we showed that H_2S treatment was able to attenuate glomerular endotheliosis in non-pregnant rats with high sFlt1.¹³ This attenuation was associated with increased renal *Vegfa* mRNA expression.¹³ In the current study, we found no up regulation of *Vegfa* gene expression in the kidneys of the NaHS treated pregnant sFlt1 rats, which might explain the absence of an attenuating effect of NaHS on the glomerular lesions. Possibly, NaHS may have different effects on renal *VEGF* gene expression during pregnancy as compared to non-pregnant rats. Moreover, in the present study we used one daily injection with 100 $\mu\text{mol/kg}$ NaHS, while previously 50 $\mu\text{mol/kg}$ NaHS was injected twice daily. Maybe the concentration of systemic H_2S was less constant during the day in the present study, reducing the potential of H_2S to achieve beneficial effects.

The glomerular lesions in the sFlt1 overexpressing pregnant rats were associated with an inflammation and profibrotic injury in the kidney. This was accompanied by increased

ICAM-1 expression. ICAM-1 is an important molecule for infiltration of macrophages into the kidney.⁴³ and renal macrophages are thought to contribute to the onset of the pre-fibrotic lesions.⁴⁴ Therefore, it may be suggested that sFlt1 increased endothelial ICAM-1 expression, resulting in macrophage infiltration, which then induced prefibrotic lesions. This is in accordance with another study in which VEGF administration led to decreased renal ICAM-1 expression and reduced macrophage infiltration in rats.⁴³ Interestingly, ICAM-1 expression tended to be lower in sFlt1-rats treated with H₂S, which is in line with previous literature.⁴⁵ It is therefore tempting to hypothesize that H₂S attenuated endothelial cell activation / dysfunction in the kidney.

Whereas the increased number of macrophages in the renal interstitium of sFlt1 rats appeared to be mainly M1 macrophages, M1 and M2 macrophages seemed to contribute more equally to the increased macrophage number in the glomeruli. In the kidney, M1 macrophages are considered to have a pro-inflammatory phenotype, and M2 macrophages are known for their tissue-repairing, but also prefibrotic properties.⁴⁴ This may explain why, in the present study, the extent of prefibrotic changes appeared to be higher in the glomeruli of the sFlt1-rats, as compared to the interstitium of these rats. It is unclear what drove the transition to, or initial influx of M2 macrophages in the glomeruli of the rats with sFlt1. Additional research, for instance on cytokine expression is needed to clarify this. The sFlt1 rats treated with NaHS showed a similar increase in renal macrophages. As H₂S is thought to have anti-inflammatory activity and it was shown by others that H₂S is able to shift M1 macrophages towards the M2 phenotype,³⁴ the lack of an effect of NaHS on the macrophages is notable.

High levels of sFlt1 have been shown to induce endothelial cell dysfunction. In the current study, with high plasma sFlt1 during pregnancy, we found no impaired endothelial-dependent vasorelaxation in the thoracic aorta of the rats. The role of the various endothelial derived vasoactive active factors, such as NO, PG and EDHF, was also not affected by high levels of sFlt1. This may be in accordance with the fact that we found no hypertension in the present study. However, hypertension is thought to be associated with impaired endothelial-dependent vasorelaxation in resistant arteries and not within the aorta. Thus, future studies to confirm the absence of endothelial dysfunction in the present model should be performed in resistance arteries. We observed no beneficial effect of NaHS on vasorelaxation *ex vivo*. However, it is difficult to indicate whether the lack of an H₂S effect is due to the absence of impaired endothelial-dependent vasorelaxation or to another factor.

Although sFlt1 or NaHS did not affect endothelial aortic vasodilation to acetylcholine, we observed an increased aortic angiotensin II sensitivity in rats with high sFlt1 as compared to control pregnant rats. This may be due to a lack of free VEGF, since Kappers *et al.*, described that the VEGFR inhibitor Sunitinib increased responsiveness to angiotensin II.⁴⁷

Our data are also in line with a previous report showing increased sensitivity to angiotensin II in pregnant mice that received an adenovirus expressing sFlt1.⁴⁸ Interestingly, in our study, treatment with NaHS seemed to reduce the sensitivity for angiotensin II towards normal levels. The present study also showed that, although not significant, an increased angiotensin II response was induced by high levels of sFlt1, which was decreased by NaHS treatment after blocking the AT2-R. This may indicate that the altered angiotensin II sensitivity is partly regulated by the AT1-R. Although others have shown that H₂S is able to down regulate tissue AT1-R expression,^{49,50} we did not observe this in the aorta (nor in the kidney). This suggests that the differences in AT1-R expression may not underlie the effect of NaHS in the present study. As H₂S has been shown to be able to decrease the binding affinity of angiotensin II to the AT1-R receptor,⁵¹ such an effect of NaHS could (partly) explain the effects in our study.

In the present study, angiotensin II induced vasoconstriction via the AT2-R, instead of vasodilation. Such a vasoconstrictor effect of the AT2-R has been observed before in (spontaneous) hypertensive rats.^{32,52,53} This may be the effect of hypertension, since lowering blood pressure restored the vasodilating effect of the AT2-R.⁵⁴ Interestingly, in our study, even in control pregnant rats, we found a vasoconstrictor, rather than a vasodilator effect of the AT2-R during pregnancy. This appeared to be a pregnancy effect, since in non-pregnant rats no vasoconstrictor response of the AT2-R on angiotensin II stimulation was observed (data not shown). The mechanism of the change in AT2-R function is still unclear, but the presence of endothelial dysfunction is proposed to be involved.³² Therefore, our data might indicate that pregnant SD rats show some endothelial dysfunction, which is in line with the suggestion that SD have vascular dysfunction as compared to Wistar rats, and are at increased risk for vascular disease upon ageing.⁵⁵ Moreover, in a meta-analysis by van Drongelen, *et al.* it was observed that SD rats retain extra mechanisms to meet the same degree of vascular adaptation to pregnancy, as compared to Wistar rats.⁵⁵ Although NaHS treatment was not able to intervene with the AT2-R function, we revealed that NaHS is able to increase *At2r* gene expression in the kidney. Hence, the effects of NaHS on the angiotensin II receptors seemed to be tissue dependent. As *At2r* gene expression may be directly down regulated by reactive oxygen species,⁵⁶ it may be suggested that

the ability of H_2S to scavenge reactive oxygen species might explain the observed effect in the kidney.

In summary, the model used in the current study was not suitable to investigate the therapeutic potential of NaHS on blood pressure and proteinuria in experimental preeclampsia, since the pregnant rats did not develop proteinuria or hypertension despite the high sFlt1 levels in our experimental setting. Therefore, we were not able to answer the main question of our study whether NaHS lowered blood pressure and proteinuria in pregnant rats. Nevertheless, the model is suitable to study other effects of NaHS *in vivo*. However, NaHS did not affect the high sFlt1-induced glomerular lesions in pregnant rats. Whether this is due to a lack of NaHS induced increase of VEGF or a lack of direct effects of NaHS on glomerular lesions remains to be investigated. Furthermore, we demonstrated that NaHS treatment in pregnant rats with high sFlt1 reduced the sensitivity for angiotensin II. As this is a major feature of human preeclampsia, this might be an interesting target for NaHS therapy to further study in the future. Moreover, the current data suggests that NaHS therapy is not harmful to rat pregnancy, implying that NaHS treatment might have beneficial effect on placental perfusion. In the future, additional studies investigating the therapeutic effects of H_2S in more suitable models for experimental preeclampsia are warranted, before proceeding with human trials.

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